IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	: 10/520,470
Applicant	: Tuschl et al
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Examiner	: Dana H. Shin
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RULE 1.132 DECLARATION

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Thomas Tuschl, declare as follows:

1. That, I am one of the inventors of the subject matter described and claimed in the United States Patent Application Serial No. 10/520,470, filed on January 7, 2005, entitled "RNA Interference By Single Stranded RNA Molecules".

2. That the publication by Tijsterman et al. (Science, January 2002, 295:694-697) entitled "RNA Helicase MUT-14-Dependent Gene Silencing Triggered in *C. elegans* by Short Antisense RNAs", discloses only post transcriptional gene silencing in *C. elegans* which was not expected to be predictive of results in mammalian cells in vitro or in vivo. *C. elegans* and plants are special in their RNAi mechanisms as they require RNA-dependent RNA polymerase (RdRP) genes which are involved in amplifying the trigger dsRNA, for this process. These genes are absent from *Drosophila* and mammalian cells.

In addition in *Drosophila* and mammalian cells, RNAi is of transient nature and restricted to cells that received an RNAi trigger. In *C. elegans* and plants, silencing signals can spread throughout the entire organism. In view of these differences in the biology and mechanism of RNAi among different species, one skilled in the art would not predict, based on studies in *C. elegans* that silencing may also be detectable in mammalian cell systems. Tuschl, Zamore, Lehmann, Sharp, and Bartel, Genes & Development, 1999, showed that long single-stranded RNA, in

contrast to long dsRNA, was not a trigger of RNAi in *Drosophila* cell lysates. In view of Tijsterman's disclosure, one could speculate that the introduction of the antisense molecule led first to RdRP-dependent dsRNA synthesis, an event that would be absent in mammals. Thereafter, regular RNAi would follow. The fact that antisense molecules trigger RNAi in rde-1 and 4 mutants would be consistent with this as these genes are required for long dsRNA triggered RNAi and RdRP mutants were never tested. This is supported by the disclosure in Tijsterman which states:

> "So where do these asRNAs fit in the RNAi pathway? One possibility is that the first step of RNAi (that is, long dsRNA diced into siRNAs) is bypassed by the administration of antisense oligomers. This predicts that asRNAs will also bypass the requirement for DCR-1, the protein that fulfills this function in RNAi (1 2 , 18-20). However, we failed to observe silencing of germline-expressed GFP in dcr-1 animals that were injected with GFP asRNAs (Fig. 1F). Furthermore, if the asRNAs were to function as siRNAs, they should be incorporated in a multicomponent nuclease, designated RISC (3), that degrades homologous mRNAs. Recently, using a Drosophila cell-free system, it was found that synthetic ds-siRNAs lead to RISC (21) but only if they are of the right size: 20 to 23 nt but not longer (4, 22). We found that 15- and 18-nt asRNAs were ineffective; but asRNAs of 22 nt and longer, up to 40 nt, were fully active to trigger gene silencing in C. elegans (Fig. 1E), suggesting that these asRNA molecules are taking another route to silence gene expression.

> An alternative explanation is that asRNAs prime RNA synthesis on the mRNA, thus resulting in dsRNA that might then be a substrate for DICER-dependent degradation. This would explain why a broad range of asRNAs (22 to 40 nt long) is proficient in triggering gene silencing and why efficient gene silencing depends on the temporal coexistence of substrate and target. In favor of this idea, we observed that modifying the 3' end of the asRNAs to prohibit polymerase action reduces the efficiency of gene silencing severely (Fig. 1E). The helicase activity of MUT-14 might thus act to permit de novo RNA synthesis on the target. Indeed, putative RNA-dependent RNA polymerases (RdRPs) are involved in RNAi and posttranscriptional gene silencing (PTGS) (23-26). In addition, biochemical and genetic support for RdRP action in amplifying the RNAi response was recently obtained (11, 27). In C. elegans, ego-1 is required for RNAi of germ line-expressed genes (23); unfortunately, we could not address the role of EGO-1 directly because of sterility and strongly disrupted gonads in ego-1 mutants."

3. That in view of the differences between nematode/plant and fly/mammals, one would not expect Tijsterman's disclosure to be predictive of results in mammalian cells. This is further supported by other publications such as:

- Mol Cell. 2002 Sep;10(3):537-48, which provides evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways

- Schwarz DS, Hutvágner G, Haley B, Zamore PD, which indicates that in Drosophila, two features of small interfering RNA (siRNA) structure--5' phosphates and 3' hydroxyls--are essential for RNA interference (RNAi). As in Drosophila, a 5' phosphate is required for siRNA function in human HeLa cells. In contrast, no evidence was found in flies or humans for a role in RNAi for the siRNA 3' hydroxyl group. In vitro data suggests that in both flies and mammals, each siRNA guides endonucleolytic cleavage of the target RNA at a single site.

The underlying mechanism of RNAi is conserved between flies and mammals and RNA-dependent RNA polymerases are not required for RNAi in these organisms.

4. That Elbashir (Nature 2001, 411:494-498) does not suggest or disclose a distribution of RNAi beyond the cellular walls. In later transfection experiments of cell cultures, only the transfected cells not the untransfected cells showed a knock-down, although the untransfected cells were directly located beside the transfected cells.

5. That based on the literature available at the priority date of the present application, the skilled person would have concluded that it would have been hopeless to use short single-stranded RNA molecules for RNAi in mammalian systems since the mechanism of amplification is lacking.

6. That it was evident that in *Drosophila* systems (e.g. *Drosophila* lysates) an active single-strand nuclease does occur which immediately degrades single-stranded RNA if it is not protected against nuclease with a GpppG cap structure. Short 5' phosphorylated RNA would be immediately degraded. Surprisingly, it was found in the present application that lysates from mammalian cells are different insofar that single-stranded RNAi is possible.

6. The undersigned further declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

by

Thomas Tuschl Sept. 12, 2008

Dr. Thomas Tuschl Date